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A STUDY OF MORPHOLOGICAL MUTANTS IN
NEUROSPORA CRASSA

THESE

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pour obtenir le grade de docteur ès Sciences Biologiques

par

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TABLE OF CONTENTS

GENERAL INTRODUCTION

PART I

I. INTRODUCTION	2
II. MATERIALS AND METHODS	
A. Strains	4
B. Media	4
C. Electron Microscopy	6
III. RESULTS	
A. Effects of CA-Media on Several Strains	7
B. "Biscuit" and "Amycelial"	9
C. Electron Microscopy of "Amycelial"	19
IV. DISCUSSION	21

PART II

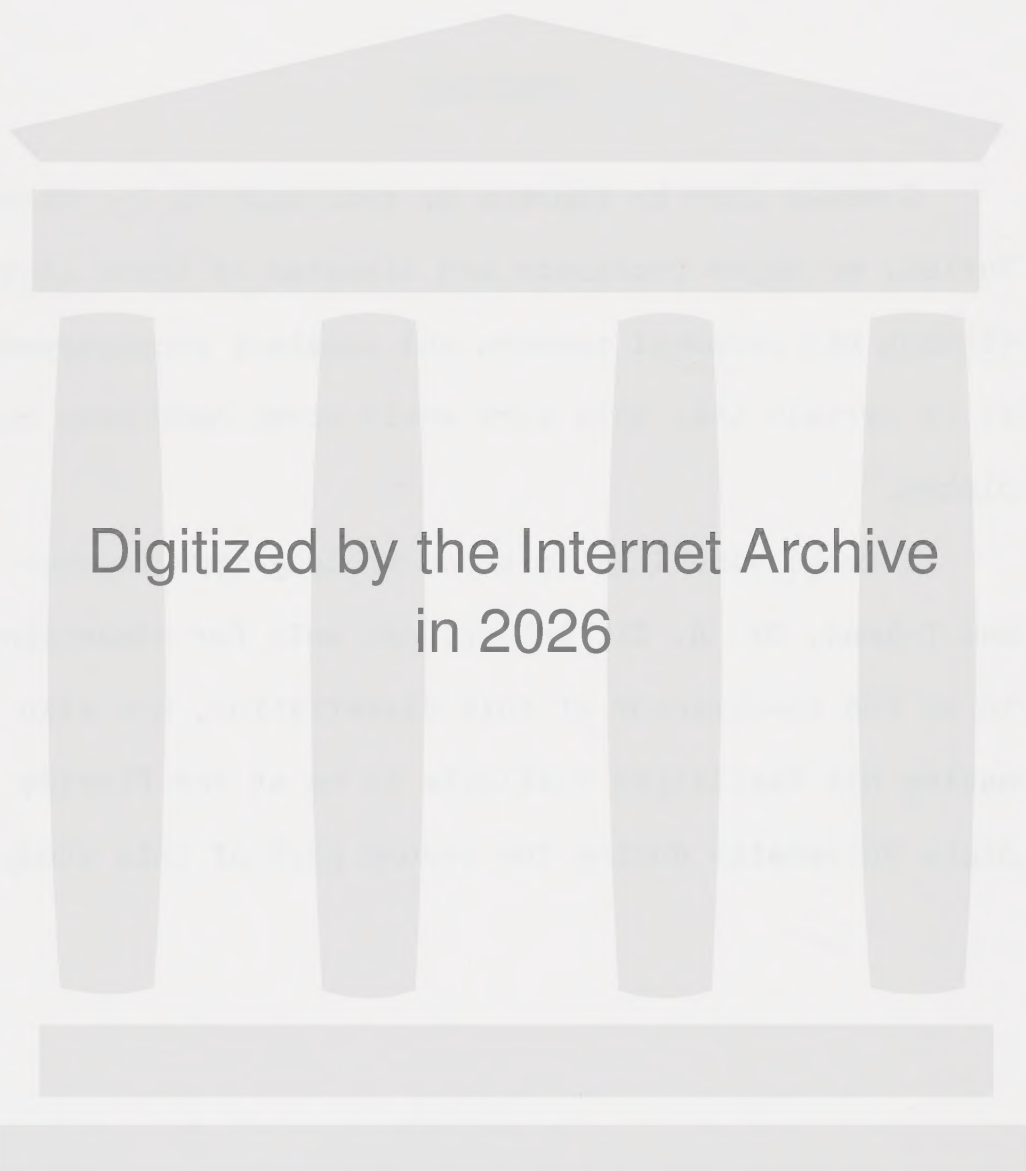
I. INTRODUCTION	25
II. MATERIALS AND METHODS	
A. Strains	29
B. Media	29
C. Analysis of the Free Amino Acid Pools	31
D. Screening Technique for Morphological Mutants	32

E.	Respiration Studies	33
F.	Partial Characterization of an Unknown Polymer Released by "Biscuit" and "Amycelial"	34
III.	RESULTS	
A.	Free Amino Acid Pools in <u>Neurospora</u>	36
B.	Modification of Morphology Using Acetate Media with Certain Amino Acids	40
C.	A Nutritional Method for the Isolation of Morphological Mutants	42
D.	Studies of Respiration	45
E.	Partial Characterization of an Unknown Poly- mer Released into the Growth Medium of "Biscuit" and "Amycelial"	52
IV.	DISCUSSION	55
V.	SUMMARY	62
VI.	REFERENCES	67

FOREWORD

I would like to express my gratitude to Dr. Gilbert Turian, my major professor and director of these studies. Without his personal concern and constant encouragement, it is certain that this work would never have been completed.

I would also like to thank my long-time teacher and friend, Dr. A. Gib DeBusk, not only for consenting to be the co-director of this dissertation, but also for making his facilities available to me at the Florida State University during the second part of this study.



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To my wife Rita

GENERAL INTRODUCTION

Morphological mutants of Neurospora crassa have been known for many years (Lindegren and Lindgren, 1941). So far they have been located at about eighty different chromosomal sites (Garnjobst and Tatum, 1967). In spite of their acknowledged value to the understanding of morphological processes in fungi, very little of the underlying biochemical basis responsible for these unusual growth patterns is understood.

In the present study, several different lines of thought and experimentation were followed in an attempt to gain a better insight into the problem.

In the early part of this work, media were developed which allowed a degree of control over certain morphological phenomena in several mutant strains of Neurospora. Observations made during the course of this first part as well as other data led to a later examination of the free amino acid pools, respiration rates, and other aspects found to differ in these mutants.

PART I

I. INTRODUCTION

Weiss and Turian (1966) demonstrated that conidogenic cultures (C-cultures) of wild type Neurospora crassa possess lower levels of pyruvate decarboxylase and ethanol dehydrogenase than does the mycelial form (M-cultures). This prompted the speculation that M-cultures predominate in fermentative enzymes whereas C-cultures possess a much lower glycolytic activity. Studies using metabolic inhibitors suggested that the process of conidiation is characteristically aerobic and is very sensitive to changes in Krebs cycle activity.

Along another line of thought, starvation conditions have often been seen to trigger morphological events leading to the production of reproductive structures. Recently Bianchi and Turian (1967) and Stine and Clark (1967) have obtained some degree of synchronization of conidiation in Neurospora utilizing essentially starvation conditions. It is a common observation that even in ordinary culture slants of Neurospora, conidiation occurs mainly at the top where the agar medium is very shallow and starvation conditions might be expected to prevail.

Young mycelia of Neurospora contain a large, constant, free amino acid pool (DeBusk and DeBusk, 1967) which could presumably be drawn on in a starvation situation and which could serve as a source of both carbon and nitrogen.

In the present study it was postulated that utilization of the endogenous amino acid pool is one of the major events during conidiation. This problem was approached by supplying amino acids exogenously as both carbon and nitrogen source. It was hoped this would produce a growing system in which the physiological events of starvation leading to conidiation would be at least partially duplicated.

II. MATERIALS AND METHODS

A. Strains

The following morphological mutants of Neurospora crassa (obtained from the Fungal Genetics Stock Center, Dartmouth College, Hanover, New Hampshire), were used in addition to a wild type strain (Lindegren A):

TABLE 1

locus	allele (isol. no.)	mating type	FGSC no.
fluffy	L	A	45
colonial-2	Y 5331	a	172
colonial-3	Y 5296	A	291
frost	B 110	A	103
colonial-4	70007	a	67
shallow	R 2371	A	88
spray	B 132	A	68
biscuit	B 6	A	277
amycelial	K 422	A	305

All of the morphological mutants were maintained on Westergaard and Mitchell (1947) minimal medium agar slants.

B. Media

For the electron microscopy, cultures were grown on media prepared using the basic salts mixture of Westergaard and Mitchell (1947) minimal medium with or without 0.2% KNO_3 depending on the case. For the rest of the experiments this mixture was modified by tripling the KH_2PO_4 concentration and

increasing that of NaCl thirty times. The NaCl increase was necessary in cases where synthetic mixtures of amino acids were used or the agar would not solidify. KH_2PO_4 concentration was increased for buffering capacity. The modified salts mixture contained the following composition per liter of medium: KH_2PO_4 , 3.0 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5g; NaCl, 3.0g; $\text{CaCl}_2 \cdot 2 \text{H}_2\text{O}$, 0.1g. Minimal media were made by supplementing either mixture with 0.2% (w/v) KNO_3 and 2% (w/v) sucrose or other carbon source. Casamino acids (Difco vitamin-free, acid-hydrolyzed casein) were added in place of KNO_3 and sucrose to make the "casamino acids medium" (CA-medium). Concentrations of the casamino acids ranged from 1% to 16% (w/v), but routinely 4% to 8% (w/v) produced the maximal effect. In all cases, pH was adjusted to approximately 6.0 after all additions had been made. Difco Bacto-Agar (2% w/v) was the solidifying agent. Synthetic mixtures of amino acids replaced the casamino acids mixture in certain instances and were prepared according to published figures for casein (McMeekin and Polis, 1949) and for the free amino acid pools of mycelia and conidia of wild type Neurospora crassa (DeBusk and DeBusk, 1967). The various media were prepared individually in flasks containing a total volume of 25 ml medium

and poured into Petri dishes. Inoculations were taken directly from agar culture slants 1-2 weeks old and the cultures were incubated at 25 degrees Centigrade.

C. Electron Microscopy

Material for electron microscopy was washed twice in distilled water and then fixed either in phosphate-buffered KMnO_4 (2% w/v, pH 7.4) for two hours at room temperature or in 6% (w/v) glutaraldehyde and subjected to a post-fixation with 1% (w/v) OsO_4 in phosphate buffer at pH 7.4 for two hours. The resulting material was dehydrated in a graded series of cold acetone to a concentration of 75% (v/v). At this point, the cells were stained for 12 hours in a 1% solution of uranyl nitrate in 75% acetone. All the material fixed with KMnO_4 and OsO_4 was taken through 100% acetone, propylene oxide, and then embedded in Epon 812. In the case of the OsO_4 -fixed cells, a staining was done using lead citrate (Karnowsky, 1961). The microscope used is a Hitachi model HS-7S.

III. RESULTS

A. Effects of CA-Media on Several Strains

Wild type Neurospora, on solid minimal medium, grows vegetatively until the mycelial front has reached the periphery of the Petri dish or flask. Growth then continues up the sides where conidiation occurs, forming a ring which expands inward as aerial growth is added. With CA(1%)-medium (1% casamino acids, w/v), however, a different pattern was seen. Mycelia behind the advancing front often underwent constrictions, forming long chains of conidia (Fig. 6), directly on the surface of the agar medium. Aerial growth then gave rise to small "clumps" of conidia ultimately giving a pebbled appearance to the entire colony. When sucrose (2%, w/v) was added to the CA-medium, the effect of the casamino acids was suppressed and the growth pattern was indistinguishable from that seen on minimal medium.

A number of morphological mutants were screened on CA-media ranging in concentrations from 1% to 16% casamino acids. Because of the difficulty and subjectivity involved in describing adequately subtle modifications, only cases where the differences were actually qualitative were chosen for work beyond this stage. Of the strains used in this

study, only "biscuit" and "amycelial" satisfied this criterion completely. Others, however, showed interesting effects and will be mentioned briefly. In general (except for "amyc"), each case where the CA-media were seen to evoke a morphological response, the addition of 2% sucrose (or several other sugars) abolished that response and the respective mutant phenotype resulted.

The morphological mutant "spray" grows on minimal medium characteristically in fan-like projections with a great deal of branching. Conidiation occurs quite late and then only on the ends of the hyphae climbing the sides of the Petri plate. The conidia are often more square in their appearance than are wild type conidia. On CA(2%-8%)-media, mycelial branching is considerably reduced and normal appearing, oval conidia are produced on aerial hyphae starting at 2-3 days when the colony diameter is still quite small.

The mutant phenotype of the strain "shallow" exhibits a dense mycelial network with a great deal of apparent intermycelial fusing. This strain also conidiates late. On CA(8%)-medium the intermycelial fusing is not seen, conidiation occurs normally, and the resulting colony is very similar to wild type growing on minimal medium.

Colonial-4 is a semicolonial mutant which normally produces conidia in large, dense clumps or balls. Here again the effect of the CA-media is apparently to correct, to an extent, the disturbance caused by the mutation so that a more nearly wild type appearance results. Interestingly, if this strain is placed on a medium containing 1% (w/v) casein (not casamino acids), as sole source of carbon and nitrogen, a non-conidiating growth pattern not unlike the "spray" phenotype is seen.

The microconidiating strain "fluffy" responds to CA(1%)-medium with an increased production of microconidia. Using higher concentrations of casamino acids however, even this difference is not seen.

The non-conidiating, highly branched morphological mutant "frost" responds to CA(4%)-medium with a modified pattern of branching and better growth. The colony however, remains non-conidiating.

The mutants colonial-2 and colonial-3 showed no apparent response to any of the media that were used.

B. "Biscuit" and "amycelial"

The mutant "biscuit" on minimal agar medium grows colonially and typically presents the type of mycelial

front seen in Fig. 1. Behind the mycelial front, aerial growth is highly fused (Fig. 3) and slides made of these cultures for 2-3 weeks after inoculation show a purely vegetative growth (Fig. 4). On CA(4%-8%)-media, mycelial branching is considerably modified (Fig. 2) and well-formed conidia are present three days after inoculation (see Fig. 5). By one week, the CA-medium-grown culture covers the entire surface of the agar and is highly conidiated while the colony on minimal medium measures only a few cm and is completely aconidial. This mutant was reported originally (Perkins, 1959) to be a conidiating strain. This was confirmed in agar slants of minimal medium where often at the very top, conidia were produced.

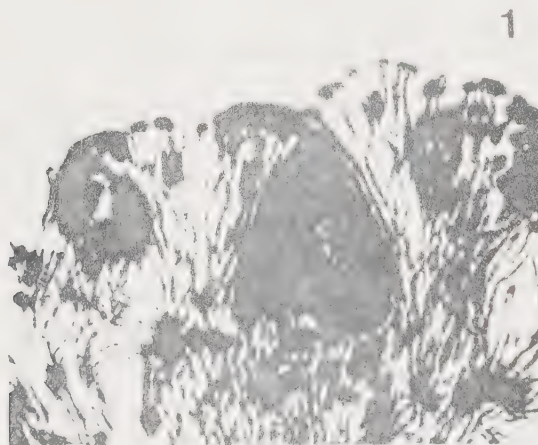


Fig. 1. Mycelial front of "biscuit" growing on minimal medium (sucrose 2%). Unstained material photographed directly on the agar by removing the top of the Petri dish. x 165

2



Fig. 2. Mycelial front of "biscuit" growing on CA(8%)-medium. Same method of preparation of the material as for Fig. 1. x 125

The same medium in a Petri dish however, centrally inoculated, produced the aconidial growth described above.

To be certain that the morphological alterations of "biscuit" seen on the CA-media were actually due to the amino acids and not to some impurities, synthetic mixtures of amino acids were prepared according to published figures for casein and the mycelial and conidial free amino acid pools of Neurospora. Technical difficulties arose using the casein mixture however, since it was difficult to dissolve the synthetic mixture in the quantities required to produce the effect (4%). This problem was overcome by using the mycelial and conidial synthetic amino acid pool mixtures (see methods), both of which produced the alterations at 1% to 2%. The casein mixture could be made to

work however, by modifying the serine and alanine portions (both of which are very low), so that they more closely

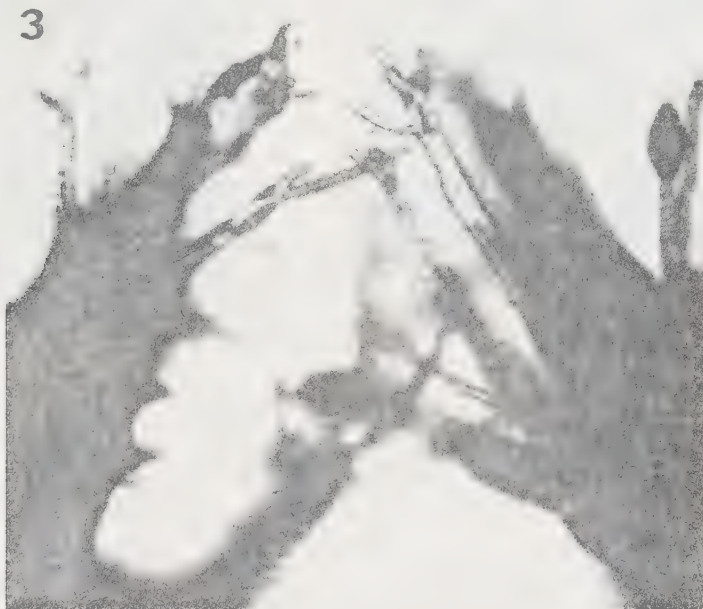


Fig. 3. Fused aerial growth behind the mycelial front of "biscuit" growing on minimal medium (sucrose 2%). The small swelling seen in the photo is a water droplet. Same method of preparation of the material as for Fig. 1. x330

resembled those found in the Neurospora pools. Use of the Neurospora pool mixtures, moreover, afforded a method by which the induction of conidiation could be studied using liquid cultures. Previously it had been observed that neither "biscuit" or "amycelial" would grow in liquid versions of the CA-media.

The colonial mutant "amycelial", so-called for its

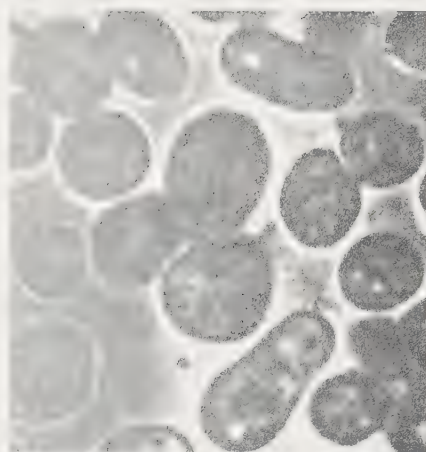
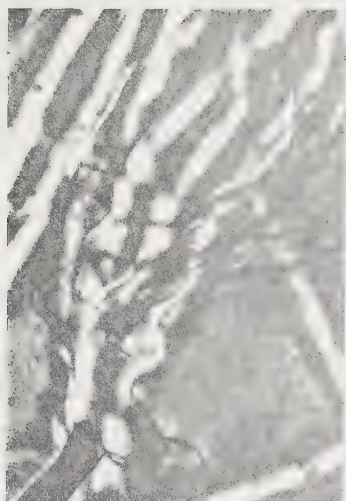


Fig. 4. An example taken from an older "biscuit" culture (minimal medium, sucrose 2%), showing vegetative growth only. In vivo slide. x 985

Fig. 5. Shows conidia harvested at about one week from a "biscuit" colony which was growing on CA(8%)-medium. In vivo slide. x 1,650



Fig. 6. Photo shows the pattern displayed by wild type cultures growing on CA(1%)-medium. Same method of preparation of the material as for Fig. 1. x 400

pseudo-mycelial structure (Fig. 10), also responds quite well to CA(4%-8%)-media. This strain, normally aconidial (macroconidialess by aschizogeny), responds with the production of conidia (Fig. 11) and displays mycelia which are much less anomalous in their appearance. The colonial characteristic however is largely retained.

Both "amycelial" and "biscuit" exhibited pigment changes in their conversions to conidiating cultures. "Amycelial" colonies are normally "lemon-yellow" while those of "biscuit" are pinkish. Both strains growing on CA-media produced the orange pigmentation usually associated with conidiation in other strains. Conidia from these two induced systems grew with their respective mutant phenotypes when reinoculations were made on minimal medium.

In an attempt to further characterize the "amycelial" and "biscuit" strains, a number of minimal media utilizing different carbon sources were prepared. Each was also tested for its ability to negate or otherwise affect the casamino acids response described above. The results seen in 7-day old cultures are summarized in Table 2.

TABLE 2

Morphological effects on "amycelial" and "biscuit" of various carbon sources both alone and in conjunction with casamino acids.

The concentration of pyruvate, as well as all of the sugars except glycerol (1% w/v) was 2% (w/v). Acetate and Krebs cycle intermediates were present at 1% (w/v) concentrations except for isocitrate and malate (2% because DL-forms were used).

Symbols: 1, growth very poor; 2, growth fair; 3, growth good; -, complete absence of conidia; *, conidia very rare but qualitatively present; **, fair conidiation; ***, good conidiation: p, proto-perithecia present; m, microconidia; I, colony phenotype intermediate between the two extremes; #, late appearance (12 days).

The basic salts mixture of Westergaard and Mitchell, modified as described earlier, was used as the basis for these media.

TABLE 2. Morphological effects on "amycelial" and "biscuit" of various carbon sources both alone and in conjunction with casamino acids

carbon source	"Amycelial"				"Biscuit"			
	Min. &		growth	CA	Min.&		growth	CA
	0.2%	KNO ₃			0.2%	KNO ₃		
none	0	0	2	**	0	0	3	***
arabinose	*	1	2	**	-p	1	3	***
lactose	-p	2	3	***	*p	2	3	***
melibiose	-p	2	3	***	*pm	1	2	**I
maltose	-	3	3	***	-I	3	3	**I
raffinose	-	2	3	***	*I	3	3	**I
starch	-	3	3	***	-p	2	3	**I
xylose	-	3	3	***	-	3	3	-
trehalose	-	3	3	***	-p	3	3	-
glucose	-	3	3	***	-	3	3	-
mannose	-	3	3	***	-	3	3	-
fructose	-	3	3	***	-	3	3	-
sucrose	-	3	3	***	-	3	3	-
glycerol	-	2	3	**#I	-	2	2	-
pyruvate(Na)	-	1	2	***	-pm	1	3	***
acetate(Na)	**#	2	3	***	-p	2	3	***
citrate	*	1	1	**	**m	1	2	***
DL-iso-								
citrate(3Na)	-	1	2	**	**m	2	2	***
a-keto-								
glutarate	-	1	2	**	*pm	2	3	***
succinate(2Na)	*#	1	2	**	**pm	2	3	***
fumarate	-	1	2	**	-p	1	3	***
malate(DL)	*#	1	2	**	*pm	2	3	***



Fig. 7. A section through a hyphal element from a culture of "amycelial" 5 days old which was grown on minimal medium (2% sucrose). Symbols: cw cell wall; er endoplasmic reticulum; lb lipid bodies; m mitochondria; N nucleus. Glutaraldehyde plus OsO_4 fixation, lead citrate staining. x 20,000

With the possible exception of glycerol none of the added carbon sources negated the casamino acids effect in "amycelial". Actually in certain cases conidiation, as well as growth, was considerably enhanced. With "biscuit" however three different classes were obtained: (1) no effect (as seen by the failure of lactose or arabinose to modify the casamino acids effect); (2) partial reversion to an intermediate phenotype (maltose, starch); (3)

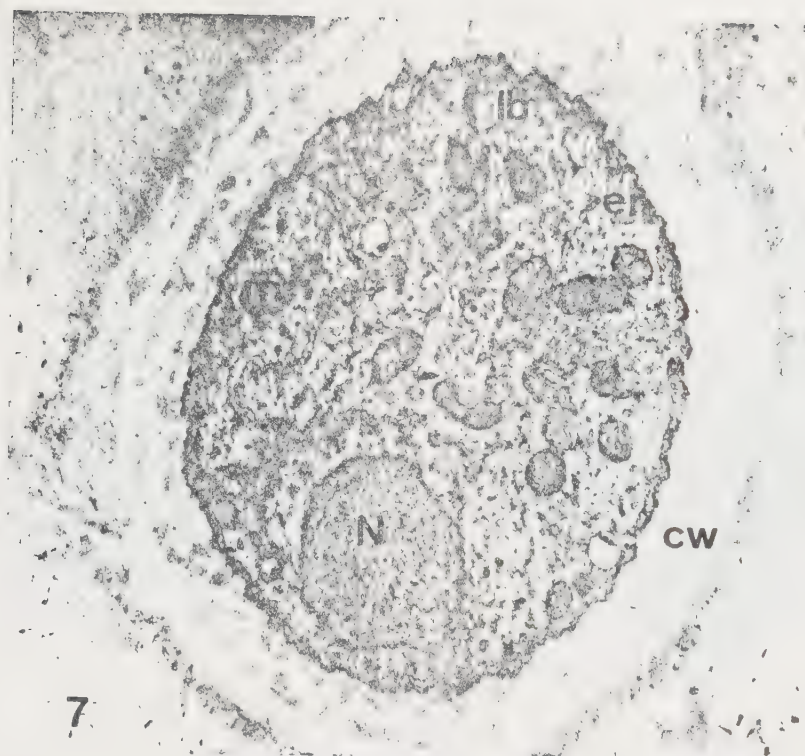


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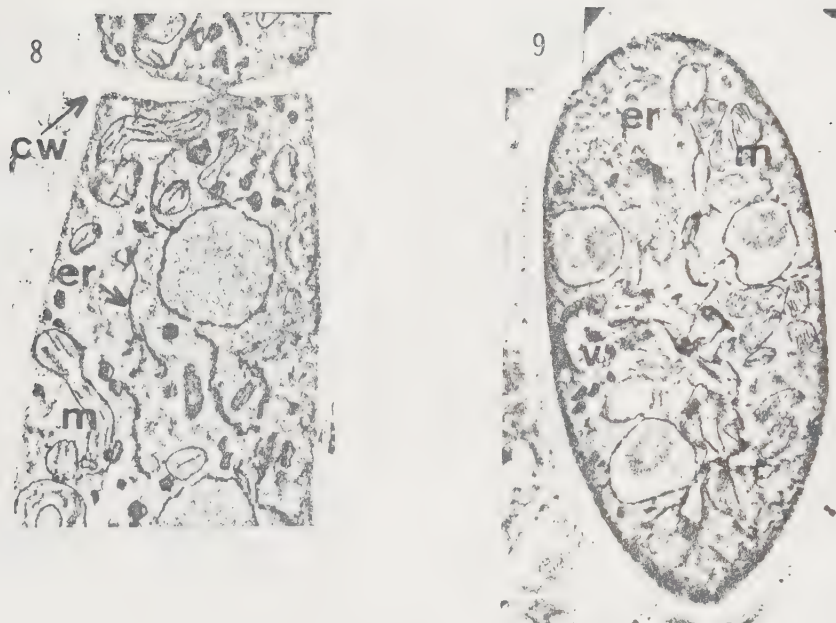


Fig. 8. Hyphal section taken at 5 days from "amycelial" growing on minimal medium with 2% acetate as carbon source. At this time the colony was completely aconidial. Note the endoplasmic reticulum (er) residing internally, thin cell wall (cw) and mitochondria (m) which are structurally well-developed. KMnO_4 fixation. x 14,000

Fig. 9. Section of an "amycelial" conidium from an 8-day culture grown on CA(8%)-medium. Note the comparatively thin cell wall, fragmented endoplasmic reticulum (er) and more highly developed mitochondria (m). v vacuole. KMnO_4 fixation. x 12,000

virtually full reversion to the mutant phenotype (glucose, fructose). Interestingly, in the last category the pigmentation characteristic failed to revert and the pigmentation remained orange even though there were no conidia.

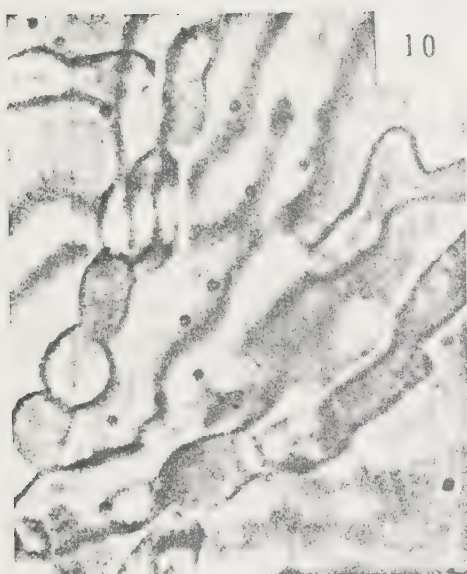


Fig. 10. Photo of a slide made from an "amycelial" culture growing on minimal medium (2% sucrose), showing "amycelial" phenotype. This colony was grown from conidia taken from CA-medium. In vivo. x 1,500



Fig. 11. "Amycelial" conidia (mainly arthrospores, one budding element on the right) harvested from CA(8%)-medium and photographed in vivo on slide. x 800

In no case was either pyruvate, acetate, or a Krebs cycle intermediate seen to cause a reversion or to interfere with conidiation. "Amycelial" grown on intermediates of the Krebs cycle or acetate grew weakly but eventually several of these gave rise to small numbers of conidia. The same phenomenon was seen more clearly using the "biscuit" strain where the production of conidia was often accompanied by the appearance of protoperithecia and microconidia.

C. Electron Microscopy of "Amycelial"

Since previous work had been done using this strain in its normal state (Oulevey-Matikian and Turian, 1968; Turian and Oulevey, 1968), it was of interest to see whether an ultrastructural basis could be found for the conversion to a conidiating phenotype. Figs. 7 and 12 demonstrate the "amycelial" phenotype, included here for the sake of continuity. Major features include an abnormally thick,



Fig. 12. A section through a hyphal element from a 5-day culture of "amycelial" growing on minimal medium (2% sucrose). This section was included because the fixation was with KMnO_4 allowing a more direct comparison with the other KMnO_4 -fixed material. The small mitochondria (m) seen in the photo are typical while the larger ones are not often seen. x 14,000

double-layered cell wall which is perhaps related to the inability of this strain to liberate free conidia ordinarily.

The endoplasmic reticulum is largely intact and usually seen in close association with the plasmalemma. The mitochondria generally are rather small with few cristae.

Fig. 8 demonstrates a mycelial section taken from a culture growing on minimal medium with acetate (2%), the carbon source. At the time this material was harvested (5 days), the colonies were still aconidial. Since these cultures eventually did produce a few conidia however, it was felt that these conditions might allow observation of an "intermediate situation". The section shows the endoplasmic reticulum still largely intact but residing more internally. At the same time (and perhaps as a result of the position of the endoplasmic reticulum), a very thin cell wall is seen.

Fig. 9 shows a conidium which was harvested at 8 days from CA(8%)-medium. The cell wall is thin as was seen in the acetate-grown culture. The endoplasmic reticulum is in close association with the plasmalemma but is fragmented along its entire length. The mitochondria were consistently seen to be larger with more and better developed cristae.

IV. DISCUSSION

Morphological mutants of Neurospora crassa have been known for many years (Lindegren and Lindgren, 1941). So far they have been located at about 80 different chromosomal sites (Garnjobst and Tatum, 1967). An often-cited characteristic of most of these mutants is that in general, their morphology is not altered in any obvious manner by the addition of supplements or other environmental changes. It is noteworthy therefore, that by using amino acids as carbon and nitrogen source, alterations were possible in 7 out of 9 of the morphological mutants used in this study. The observation that sugars were usually seen to nullify the morphogenetic effects of the amino acids explains perhaps why this phenomenon was not observed earlier. Also pertinent might be the fact that high concentrations of casamino acids were required to produce the alterations. It is difficult to say whether the use of amino acids "per se" was the causative factor for the observed morphological alterations or whether these conditions were simply instrumental in by-passing a defect of carbohydrate metabolism. From the work of Brody and Tatum (1966), it is known that such mutations occur and can lead to changes in morphology. The fact that even the wild type strain was altered however,

indicates that both of these possibilities might occur.

Pyruvate, acetate, and Krebs cycle intermediates did not interfere with the action of the casamino acids and in certain combinations (acetate and Krebs cycle intermediates) were themselves capable of producing similar effects. This supports earlier observations on the conidiogenic properties of acetate (Turian, 1961) and suggests that the observed action of the casamino acids is at the level of the citric acid cycle. If this is true, then the free amino acid pool of Neurospora, comprising some 9% of mycelial dry weight might be looked upon as an attractive source of these intermediates capable of being drawn on under starvation conditions. Proteolysis could be expected to maintain the pool during this process. In this connection it is interesting to note that young mycelia of wild type Neurospora contain by dry weight 9% free amino acids and 49% amino acids bound in protein while conidia contain only 4% free and 28% protein-bound amino acids (DeBusk and DeBusk, 1967). In Aspergillus flavus (Pillai and Srinivasan, 1956), the concentration of mycelial protein was seen to fall by nearly 50% during sporulation. Utilization of endogenous protein and amino acids during starvation and production

of fruiting bodies has also been described in Dictyostelium discoideum (Wright, 1966).

It has been shown that transport of amino acids in Neurospora as well as the size of the amino acid pools is considerably reduced in the presence of glucose or sucrose (DeBusk and DeBusk, 1965). These authors moreover state that although there is quite a bit of variation in amino acid pools in various strains of Neurospora, some of the "exotic" strains show marked differences (DeBusk and DeBusk, 1967). It should be interesting in the future to determine whether the morphological mutants used in this study will have such an imbalance in their free amino acid pools and whether a "correction" of the pools would be seen to parallel the morphological alterations described here.

A comparative study of the morphology of "amycelial" using the electron microscope revealed modifications of the cell wall, endoplasmic reticulum, and mitochondria, accompanying an induction of conidiation using the casamino acids system. Weiss and Turian (1966) reported mitochondrial differences between conidial cultures (C-cultures) and mycelial cultures (M-cultures), of wild type Neurospora. Modifications of the cell wall were also noted. Del Vecchio and Turian (1968), using the morphological mutant "spray"

(which does not respond differentially to C and M-media), failed to see these differences. Although the cell wall changes seen in wild type differed from those seen with "amycelial", it is still noteworthy that modifications did occur. Growing on minimal medium, "amycelial" displays an endoplasmic reticulum in close association with the plasmalemma (Figs. 7 and 12), a condition seen typically in yeast growing anaerobically but not aerobically (Moor, 1967). Interestingly, "amycelial" produces large quantities of ethanol (Oulevey-Matikian and Turian, 1968), similar in that respect to the M-cultures of wild type. It is assumed that the modification of the endoplasmic reticulum accompanying the induction of conidiation in "amycelial" is somehow connected with a more aerobic type of metabolism which almost certainly accompanies this transition.

PART II

I. INTRODUCTION

DeBusk and DeBusk (1967) reported marked differences in ~~the~~ free amino acid pools of geographical isolates of Neurospora crassa. In the first part of this study, cas-amino acids were seen to produce morphological alterations in Neurospora and accordingly a series of experiments was begun to determine whether some of the morphological mutants might show abnormalities in their free amino acid pools. Since electron micrographs had demonstrated changes occurring at the level of the cell wall, mitochondria, and endoplasmic reticulum, these foci of interest were also kept in mind in the second part of this study which was approached from a more purely biochemical point of view.

Crocken and Tatum (1968) found that in the presence of sorbose (which inhibits conidiation and produces a colonial morphology in Neurospora), glucose was utilized less efficiently. Less dry weight and more carbon dioxide was produced, more oxygen was consumed per milligram of glucose used and the incorporation of labeled glucose into cell-wall polymers and into trehalose was lower. They suggest that the effects of sorbose on the growth and

morphology of Neurospora involves a general metabolic disturbance resulting from a partial uncoupling of respiration and oxidative phosphorylation.

Beadle (1944) noted that cultures of inositol-requiring mutants of Neurospora grown on low inositol levels adhere to the sides of the culture flasks and produce small, dense hyphal pellets rather than the spreading mat formed at optimal inositol levels. Continuing this work, Shatkin and Tatum (1961) obtained electron micrographs of cell fractions indicating that inositol is a structural constituent of Neurospora lipoprotein membranes, including plasmalemma, nuclear envelope, mitochondrial membranes, and endoplasmic reticulum. Normal inositol-requiring and wild type hyphae are similar in ultrastructure. However, suboptimally cultured, colonial, inositolless hyphae contain large lipid droplets, and the cellular membranes are in various stages of degeneration. They suggest that the lipid droplets are products of membrane catabolism and propose that the colonial morphology of the inositol-requiring mutant of Neurospora is due to a metabolic imbalance between membrane synthesis and the synthesis of other cellular constituents.

The relationship of cell wall composition to morphology in Neurospora was studied by Mahadevan and Tatum, (1965)

by correlating the levels of structural polymers of the cell wall with wild type and colonial morphology. They found the levels of one or more of these structural polymers to be consistently altered in single gene mutants with colonial morphology, and in sorbose-induced colonial growth. Particularly important to morphology appeared to be a peptide-polysaccharide complex and a B-1, 3-glucan.

Reissig (1968) has isolated a mucopolysaccharide (MP) from the culture medium of one of the colonial-temperature sensitive (COT) strains of Neurospora. COT is colonial at 35 degrees, but not at 25 degrees. Shake cultures at 35 degrees grow at the wild type rate during the first twelve hours (unrestricted phase) and then slow down several fold (restricted phase) until they reach a stationary phase. MP isolated from the stationary phase triggers restricted growth both in unrestricted 35 degree cultures and in 25 degree cultures where the restriction is more severe and accompanied by agglutination and vacuolization. Slime spheroplasts are also agglutinated, suggesting that the MP receptors are on the membrane. MP has also been seen to efficiently inhibit respiration in wild type conidia (STA₄) (DeBusk, unpublished data), thus (as in the case of sorbose

induced colonial growth) implicating respiration in some way with colonial growth.

Support for some of these diverse observations was obtained by Oulevey-Matikian and Turian (1968), as well as Dicker, et.al., (1969), who obtained electron micrographs showing alterations in the cell wall, endoplasmic reticulum, and mitochondria of the Neurospora morphological mutant "amycelial". In addition, "amycelial" was found to produce large quantities of ethanol, indicating a predominately fermentative type of metabolism, similar in that respect to the M-cultures of Turian (1964) where conidiation was suppressed by favoring this type of metabolism.

In the present study, these various data were kept in mind, and accordingly, emphasis was placed at the level of the cell walls, membranes, and mitochondria all of which are known often to show abnormalities where abnormal morphology is seen.

II. MATERIALS AND METHODS

A. Strains

The following strains of Neurospora crassa were used:

TABLE 3

locus	allele (isol. no.)	linkage group	*FGSC no.
STA ₄	(wild type)	---	262
Lindegren A	(wild type)	---	853
amycelial	K422	I	305
biscuit	B6	V	277
spray	B132	V	68
colonial-4	70007	IV	67
frost	B110	I	103
shallow	R2371	V	88
balloon	B56	II	105
plug	B118	V	96
button	B40	VII	182
skin	B234	VII	808
colonial-2	Y5331	VII	172
fluffy	L	II	45
dapple	R2375	II	1077
doily	LD-55-51	VII	177
carpet	P564	II	292
medusa	R2401	IV	1403
crisp	B122	I	804

*FGSC- Fungal Genetics Stock Center
Dartmouth College
Hanover, New Hampshire

B. Media

During the course of these experiments, media were based upon the basic salts mixture of Westergaard and Mitchell (1947) minimal fruiting medium (P-medium) or a

modification (P-mod. medium) giving the following compositions per liter of medium:

	P-medium	P-mod. medium
KH_2PO_4	1.0 g.	3.0 g.
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	0.5 g.	0.5 g.
NaCl	0.1 g.	3.0 g.
$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$	0.1 g.	0.1 g.
FeCl_3	0.005 g.	0.005 g.
biotin	50 ug.	50 ug.
*trace elements	1 ml.	1 ml.

* (Beadle and Tatum, 1945)

The various media were made by supplementing either of the above mixtures with KNO_3 (0.1% or 0.2% w/v) or other nitrogen source (single amino acids in certain experiments) and 2% w/v sucrose or other carbon source.

For the nutritional screening of morphological mutants (described below) the P-salts mixture was used, supplemented with 2% sodium acetate and 10^{-2}M glutamic or aspartic acids as sole sources of carbon and nitrogen respectively.

The mycelial (M) medium of Turian (1964) was employed in one instance as described in the text.

Casamino acids (Difco vitamin-free, acid-hydrolyzed casein) were added in place of KNO_3 and sucrose to make the "casamino acids media" (CA-media). Concentrations of the casamino acids ranged from 1% to 16% (w/v) but routinely 8% (w/v) was used for the production of conidia in non-conidiating and poorly conidiating strains. In all cases, pH was adjusted to approximately 6.0 after all additions had been made. Difco Bacto-agar (2% w/v) was the solidifying agent for the solid media. Incubations were always carried out at 25 degrees.

All of the strains were maintained on Westergaard and Mitchell minimal medium agar slants.

C. Analysis of the Free Amino Acid Pools

In measuring the conidial free amino acid pools, conidia were harvested after an appropriate growth period on solid medium into distilled water, filtered to remove mycelial fragments, and washed and centrifuged two times in distilled water. A portion was dried at 55 degrees to obtain a dry weight and the remaining suspension adjusted in volume to a concentration of 5 - 15 mg dry weight of conidia per ml. The samples were placed in a boiling

water bath for 30 minutes, centrifuged to remove the conidia, and the pH adjusted to 2.2 along with any necessary volume adjustments.

The same procedure was followed in the extraction of mycelial free amino acid pools. All samples were analyzed with a Beckman 120-B amino acid analyzer.

D. Screening Technique for Morphological Mutants

A method was developed, based largely upon the filtration enrichment technique of Catcheside (1954), for the isolation of large numbers of morphological mutants of Neurospora. In this procedure, a suspension of wild type conidia (STA_4) containing approximately 10^7 cells per ml. was irradiated with ultraviolet light to produce 85-90% kill. After a 35 minute period in the dark, (Kilby and deSerres, 1967) 5 ml. of the suspension were inoculated into a bubble tube containing 40 ml. P-medium with sodium acetate (2% w/v) and either glutamic or aspartic acid ($10^{-2}M$) as sole sources of carbon and nitrogen respectively. The pH was adjusted to 6.0. Large test tubes 27 x 200 mm. were used for incubation as well as for subsequent filtrations. The incubation mixture was bubbled in these tubes with wet, sterile air through a disposable glass transfer

pipette with its cotton plug inserted to complete the new bubble tube. Incubation was carried out at 25 degrees with filtrations being made at approximately 12, 18, and 24 hours, and then at 12 - 24 hour intervals (depending on growth) to a total incubation time of 72 hours.

After 72 hours the ungerminated conidia were concentrated either by filtration or centrifugation and re-suspended in 2 - 3 ml. sterile, distilled water. All of the cells were then spread on 10 - 15 petri plates containing solid Westergaard and Mitchell minimal medium (Ps).

The identification and isolation of the morphological mutants was carried out with a dissecting microscope as soon as possible, ordinarily 24 - 36 hours after plating. This was found to be necessary to avoid overgrowth by wild type cells surviving the filtrations. Alternatively it is possible to plate the cells on minimal sorbose agar plates but recognition of the mutant colonies under these conditions is more difficult. An average run yields approximately 20 mutants.

E. Respiration Studies

For measurements of oxygen consumption conidia were used since they could be handled more easily than mycelia.

Conidial suspensions were made at approximately 2 degrees and adjusted to 1.0 mg dry weight of conidia per ml. Each Warburg vessel contained the following components: 0.2 ml KOH (20% w/v) in the center well, 1.5 ml P-medium (with 0.1% w/v KNO_3) concentrated twice, 0.5 ml 10% (w/v) glucose or sodium acetate or 0.5 ml. distilled water (in cases where no carbon source was present), 1.0 ml conidial suspension. The conidial suspension was added last and the reaction vessels were allowed to equilibrate in the water bath (25 degrees) for 5 minutes before closing the stopcocks.

F. Partial Characterization of an Unknown Polymer Released by "Biscuit" and "Amycelial"

After an appropriate period of growth in Ps medium, the cells were filtered and ethanol added to the filtrate to a concentration of 40% (v/v). A floccose precipitate forms overnight in the cold which was centrifuged for 30 to 90 minutes at 4000 RPM (g-2520). The supernatant was discarded and the precipitate washed once with 40% (v/v) ethanol and centrifuged as before. Analyses of sugar and amino acid components of the samples were performed through the courtesy of Professor R.J. Winzler, of the State University of New York at Buffalo.

The substance released by the mutant "biscuit" was analyzed for the presence of polyphosphate. This was done by using the isolation procedures of Harold (1960) and the phosphate determination of Lowry and Lopez (1946).

III. RESULTS

A. Free Amino Acid Pools in Neurospora

Starvation conditions have often been seen to trigger morphological events leading to the production of reproductive structures. Bianchi and Turian (1967) and Stine and Clark (1967) have obtained some degree of synchronization of conidiation in Neurospora utilizing essentially starvation conditions. It is a common observation that even in ordinary culture slants of Neurospora, conidiation occurs mainly at the top where the agar medium is very shallow and starvation conditions might be expected to prevail.

Young mycelia of Neurospora contain a large, constant, free amino acid pool (DeBusk and DeBusk, 1967) comprising some 9% of mycelial dry weight which might be looked upon as an attractive source of both carbon and nitrogen, capable of being drawn on under starvation conditions. Proteolysis could be expected to maintain the pool during this process. In this connection it is interesting to note that young mycelia of wild type Neurospora contain by weight 9% free amino acids and 49% amino acids bound in protein while conidia contain only 4% free and 28% protein-bound amino acids (DeBusk and DeBusk, 1967).

Following the observations of the morphological effects brought about by the casamino acids it seemed worthwhile to follow this line of investigation by studying directly the free amino acid pools of wild type and several of the morphological mutants.

Table 4 shows gross alterations present in the free amino acid pools of many of the morphological mutants of Neurospora. With the exception of the mutant "shallow", all of the mutant strains shown here were non-conidiating in the liquid Westergaard and Mitchell minimal medium employed. In addition, wild type cultures growing in liquid M-medium of Turian (1964) (which prevents conidiation) also showed similar variations in the free amino acid pool. All of the morphological mutants showed varying quantities of an unknown ninhydrin-positive substance which was subsequently found to be located primarily in the mitochondrial fraction.

Amino acid pool analyses were performed on several strains growing in minimal medium with sodium acetate the carbon source (Pa-medium). Table 5 shows two strains, "spray" and "amycelial", aconidial in this medium and two strains, "plug" and "biscuit", which conidiate well beginning on about the third day after inoculation. Although these conditions modify the balance of amino acids compared to the sucrose-containing media, the total deficiency with

TABLE 4. The free amino acid pool composition of several strains of Neurospora crassa

TABLE 4

amino acid	FS-1		FS-1		Bis		Amyc		Sp		Col4		Sh		Bal		Bn		Sk		Col2		Pl	
	Ps-3	M-3	M-3	M-3	Ps-5	Ps-5	Ps-7	Ps-7	Ps-7	Ps-7	Ps-5	Ps-5	Ps-5	Ps-5	Ps-5	Ps-5	Ps-5	Ps-5	Ps-5	Ps-5	Ps-5	Ps-5	Ps-3	Ps-3
lys	61.4	23.7			6.0	2.3	1.4	2.3	1.4	8.6	12.8	5.0	10.8	6.3	5.0	9.3								
his	10.7	8.3			3.6	1.7	0.8	1.7	0.8	3.2	4.2	2.6	3.9	4.5	2.4	3.6								
unkn.	--	--			2.2	4.8	1.2	4.8	1.2	1.4	5.4	1.2	1.3	2.7	2.6	0.2								
arg	47.1	26.9			12.6	6.4	1.8	6.4	1.8	10.8	9.5	3.8	4.6	8.9	9.5	7.1								
%basics	18.4	20.0			9.6	5.5	4.4	5.5	4.4	12.1	6.3	8.7	17.3	7.9	8.0	9.9								
asp	4.1	3.9			3.2	--	--	--	--	2.9	7.5	11.2	8.5	12.8	8.6	6.5								
thr	17.6	9.4			5.4*	5.6	2.1	5.6	2.1	4.7	12.0	3.6	3.9	8.0	6.5	6.4								
ser	88.3	42.5			36.4	23.2	9.5	23.2	9.5	21.0	34.9	15.5	13.9	35.6	35.0	33.8								
glu	76.8	27.6			53.8	37.7	26.5	37.7	26.5	42.2	33.7	40.8	31.4	56.8	58.4	48.7								
pro	4.2	4.0			2.8	3.8	1.2	3.8	1.2	2.5	8.9	2.8	2.4	0.8	3.2	2.6								
gly	13.1	10.3			4.1	2.1	1.2	2.1	1.2	3.7	10.6	3.4	3.4	6.5	4.5	4.1								
ala	274.6	119.1			80.2	75.9	34.9	75.9	34.9	71.2	153.0	24.4	22.5	75.9	44.2	54.5								
l/2cys	27.8	5.5			8.3	12.1	5.2	12.1	5.2	4.0	25.4	6.0	--	8.7	5.6	13.8								
val	11.0	4.1			8.7	4.8	2.4	4.8	2.4	5.0	24.2	3.6	--	8.9	8.1	5.0								
met	--	--			0.9	0.5	0.3	0.5	0.3	--	2.4	0.8	--	1.5	1.7	0.4								
ileu	2.8	2.1			1.6	0.8	0.5	0.8	0.5	1.5	8.1	1.7	1.1	3.0	3.3	1.3								
leu	4.6	3.6			2.8	1.6	0.8	1.6	0.8	2.5	15.4	2.2	2.1	4.5	6.5	2.6								
tyr	1.4	1.2			0.9	0.7	0.3	0.7	0.3	1.0	6.8	1.1	0.8	1.6	2.1	1.6								
phe	1.4	1.2			1.1	0.8	0.3	0.8	0.3	1.3	6.3	0.9	0.9	1.9	3.3	1.5								
total	647	293			232	190	90	190	90	187	337	132	111	249	211	205								
% w.t.	100	45.4			35.9	29.3	14.0	29.3	14.0	29.0	60.0	20.3	17.2	38.5	32.6	31.4								

The designations at the head of each column refer respectively to the strain, the growth medium and the age of the culture in days.

The values listed are expressed as micromoles/gram dry weight.

* signifies approximate values.

TABLE 5. The free amino acid pool composition of several strains of Neurospora crassa growing in acetate-containing medium.

amino acid	Sp Pa-7	Amyc Pa-7	Pl Pa-2	Bis Pa-2
lys	2.5	2.2	17.9	20.7
his	2.0	0.8	1.6	8.0
unkn.	2.5	4.2	5.5	3.5
arg	2.3	1.6	5.9	12.5
% basics	6.1	5.5	15.2	20.0
asp	2.5	1.3	2.2	3.4
thr	4.9	4.0	4.7	3.5
ser	18.6	11.2	12.5	26.7
glu	41.8	23.2	31.6	26.4
pro	2.9	1.1	2.7	1.9*
gly	3.5	3.8	6.2	6.1
ala	12.5	19.0	52.4	68.4
1/2 cys	3.7	0.9	5.4	9.4
val	4.1	3.3	4.6	7.1
met	0.7	0.9	0.9	2.3
ileu	1.3	1.4	2.2	0.8*
leu	2.6	2.3	2.4	1.7
tyr	1.2	1.1	1.3	0.4*
phe	1.2	1.4	trace	0.3*
total	110.7	83.5	167.1	199.7
% w.t.	17.1	12.9	25.8	30.9

The designations at the head of each column refer respectively to the strain, the growth medium, and the age of the culture in days.

The values listed are expressed as micromoles/gram dry weight.

* signifies approximate values.

respect to wild type cultures is by no means corrected. An observation, perhaps of importance is the fact that most of the morphological mutants display very low values for the basic amino acids. This "imbalance" is to a large extent corrected in the two cases, "plug" and "biscuit", shown in Table 5 where conidiation was induced in the Pa-medium. This correction did not occur with "spray" and "amycelial"; both aconidial in Pa-medium.

B. Modification of Morphology Using Acetate Media with Certain Amino Acids

It was seen in the first part of this study that sodium acetate could be added to the casamino acids media without negating the effects of casamino acids on the morphological mutants. Advantage was taken of this fact; a series of experiments was begun testing the effects of single amino acids when these were used as sole sources of nitrogen in the P-salts medium supplemented with 2% sodium acetate. This allowed fair growth in the majority of cases and "forced" a utilization of the amino acid being tested.

Table 6 summarizes this work. Of the strains listed, "frost", "amycelial", and "biscuit" are completely aconidial

TABLE 6. Morphological effects of single amino acids (.01M) when used as sole sources of nitrogen in an acetate-containing medium

STRAIN AGE (DAYS)	Amc 5	Bis 5	Pl 6	Col-4 5	Fr 4	Sp 4
Amino Acid						
try	1 **	1 -	1 **	1 **	1 **	1 **
lys	2 -	2 -	2 -	2 -	2 -	2 -
his	2 **	2 **	2 **	2 **	2 -	2 *
arg	2 *	2 #	2 **	2 **	2 -	2 -
asp	2 -	0	2 **	0	0	0
thr	2 -	2 -	2 **	2 *	2 -	2 *
ser	2 -	2 -	2 **	2 **	2 -	2 *
glu	0	0	0	0	0	0
gly	2 -	2 -	2 **	2 **	2 -	2 -
ala	2 -	2 -	2 **	2 **	2 -	2 -
val	2 *	2 -	2 **	2 **	2 -	2 *
met	1 *	1 -	2 **	2 **	2 -	2 -
isoleu	2 *	2 -	2 **	2 **	2 -	2 *
leu	2 *	2 -	2 **	2 **	2 -	2 -
tyr	2 *	2 -	2 **	2 **	2 -	2 **
phe	2 *	2 -	2 **	2 **	2 -	2 -

Symbols: 0, complete absence of growth; 1, very poor growth; 2, growth fair to good; -, complete absence of conidia; *, conidia rare but present; **, good conidiation; #, good conidiation at .04 M L-arginine. The number following the strain designation indicates the age of the culture (days). The basic salts mixture of Westergaard and Mitchell, as described earlier was used as the basis for these media.

on minimal medium (Ps), "spray" conidiates poorly and late, and both "colonial-4" and "plug" conidiate well on minimal medium. From the table it can be seen that the various strains differ considerably in their responses to the different amino acids. Few generalizations can be made.

Several combinations however, gave surprizing effects. Tryptophan, along with poor growth often stimulated a precocious condition. In "frost" this was the only combination found capable of stimulating conidiation. Lysine uniformly supported fair growth but suppressed conidiation, even with "plug", and "colonial-4". Both of these later strains conidiate well even on minimal medium (Ps). Histidine stimulated conidiation in both "amycelial" and "biscuit" but was ineffective with "frost" and "spray". Peculiar results were obtained using both glutamic and aspartic acids. In the majority of cases there was a complete lack of growth. Since wild type cells grow very well on this combination, this observation was later used as a basis for the screening and isolation of many other morphological mutants of Neurospora. Of the remaining combinations it is of interest that tyrosine stimulated a strong conidiation in the mutant "spray". This combination later was used to obtain the numbers of conidia necessary for respiration studies.

C. A Nutritional Method for the Isolation of Morphological Mutants

From the previous section it was observed that many of

the morphological mutants failed to grow when glutamic or aspartic acids served as nitrogen source. Table 7 shows the growth responses of several other strains under these

TABLE 7. Growth response of several known morphological mutants to the selective media used for concentrating unknown mutants as described in the text.

Strain	Acetate- Glutamate	Acetate- Asparate
amycelial	--	**
biscuit	--	--
plug	--	**
colonial-4	--	--
fluffy	--	**
spray	--	--
frost	--	--
dapple	**	--
doily	**	--
carpet	--	--
medusa	--	--
crisp	**	**
colonial-2	**	**
STA ₄	**	**
LIND A	**	**

Symbols: **, growth
 --, absence of growth

conditions. This was particularly interesting since differences in nutritional requirements between wild type and morphological mutants previously have not been observed. This observation formed the basis for the selective screening of morphological mutants as described in the Materials

and Methods section. Figure 13 shows a few of the various colony phenotypes which were obtained by this method.

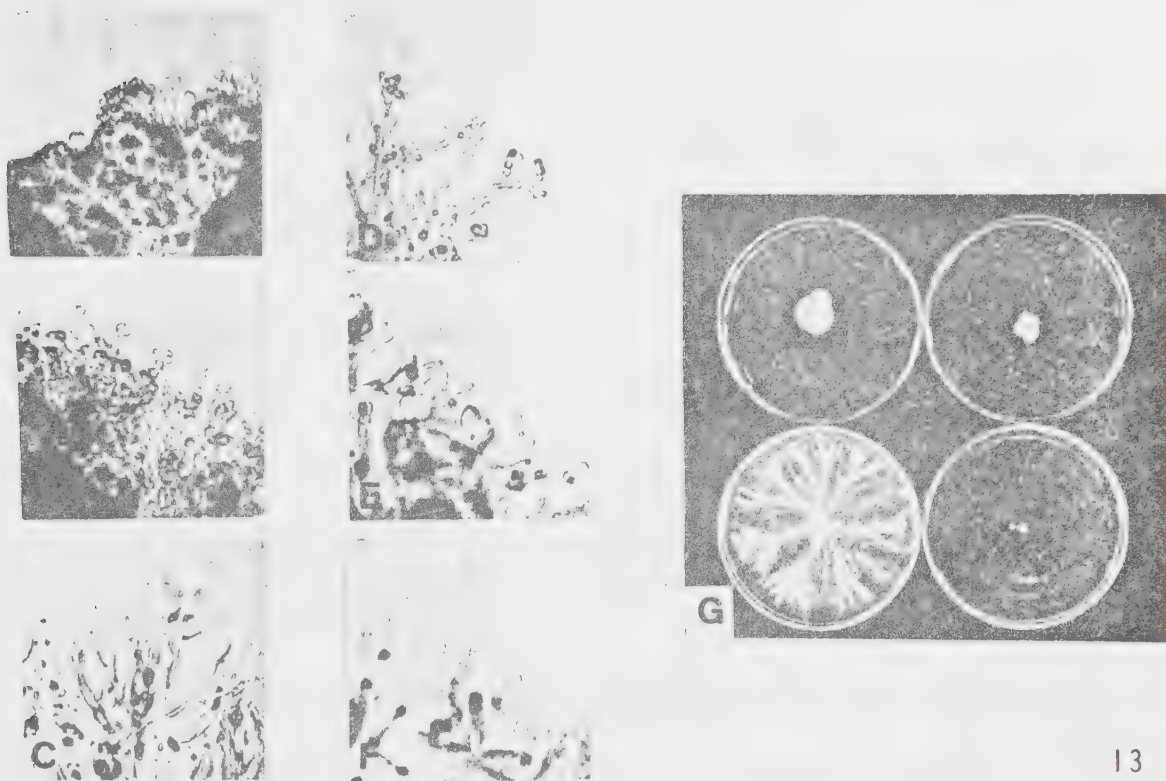


Fig. 13. Several unmapped, representative morphological mutants obtained using the nutritional screening method described in the text.

Of the underlying biochemical basis for these growth responses, very little is known. Preliminary experiments with "amycelial" and "biscuit" have shown, however, that malate or alpha ketoglutarate ($10^{-2}M$) will alleviate the inhibition seen on acetate-glutamate plates. Accompanying this growth response moreover, there is an induction

of conidiation and other morphological alterations similar to those seen in the "casamino-acids" systems. These results again stress the importance of oxidative metabolism for the maintenance of normal morphology in Neurospora and suggest a new method of grouping morphological mutants that may be helpful for future work.

D. Studies of Respiration

A study of respiration in several strains of Neurospora was begun for the following reasons:

1. Croken and Tatum's (1968) work with colonial growth induced by the presence of sorbose in the medium indicated that an uncoupling of respiration and oxidative phosphorylation might be important to morphology in Neurospora.

2. The electron micrographs of "amycelial" described earlier indicated abnormalities at the mitochondrial level in this strain.

3. High levels of ethanol produced in the medium of cultures of "amycelial" (Oulevey-Matikian and Turian, 1968) indicated a predominately fermentative type of metabolism.

4. The failure of many morphological mutants to grow on acetate-glutamate or aspartate (see Table 7) plates also

suggested difficulties at the mitochondrial level.

5. Finally, the observed imbalance of the total free amino acid pools described earlier in this paper also was indicative of abnormalities at the level of oxidative metabolism.

Since media had been developed for the induction of conidiation, conidial suspensions of several strains were used for the Warburg studies for reasons of convenience. Conidia from "amycelial" and "biscuit" were obtained as described in the Materials and Methods section. "Spray" conidia were harvested from a medium employing the P-mixture with sodium acetate (2% w/v) and L-tyrosine ($10^{-2}M$) the sole source of carbon and nitrogen respectively. This medium was developed empirically and the reason for its effectiveness is not understood at this time. Any of the other amino acids used in place of tyrosine in this combination were comparatively ineffective in the induction of conidiation.

Figure 14 shows that great differences exist in these strains with regard to oxygen consumption. "Spray" and "biscuit" conidia consume oxygen at rates two to three times that of wild type while that of "amycelial" is practically zero. Recently Turian (unpublished results) has

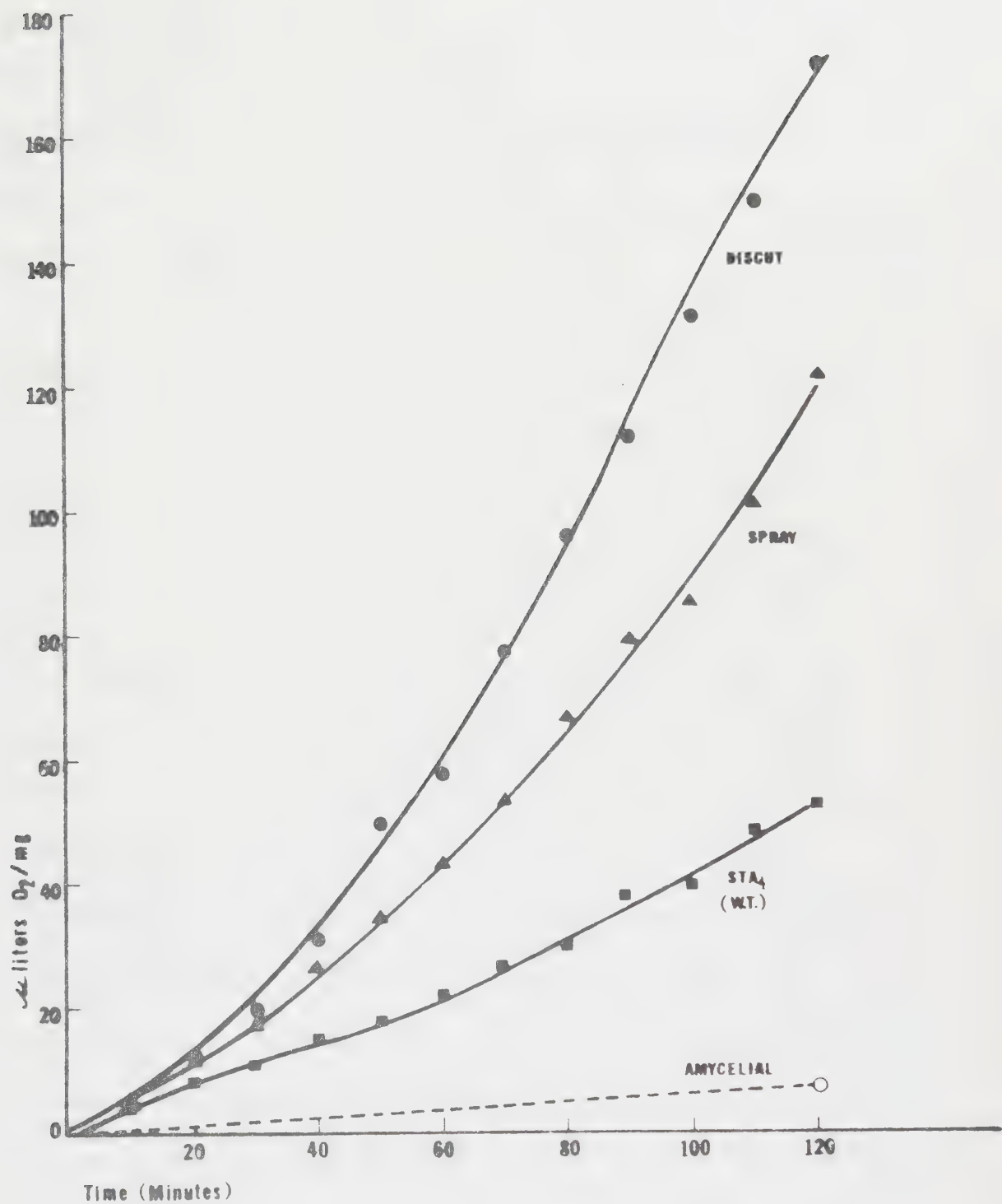


Fig. 14. Oxygen consumption of several strains of *Neurospora crassa*

All of the cultures in this figure were incubated in the presence of glucose utilizing the P-salts mixture as described in the text.

measured an alcohol dehydrogenase activity in "biscuit" even higher than that of "amycelial" thus showing that both of these strains rely largely upon fermentative processes for energy production.

Figure 15 shows the consumption of oxygen by wild type conidia incubated in an acetate medium (Pa); a glucose medium (Pg); and a medium containing neither carbon nor nitrogen (P) designed to measure the rate of endogenous respiration. It can be seen that oxygen consumption is higher in the acetate medium. This is probably due to known feedback systems linking glycolysis with oxidative phosphorylation which would be operative in the glucose but not in the acetate medium.

Figures 16 and 17 show that this regulation is apparently disturbed in conidia of the morphological mutants "biscuit" and "spray". All of the values obtained with "spray" were less extreme than either "biscuit" or "amycelial". Interestingly, "spray" is less extreme in its morphological phenotype as well (being capable of producing a few conidia on minimal medium). Cultures of "biscuit" showed a rather high endogenous respiration explaining perhaps why these conidia lose their viability after relatively short periods of storage.

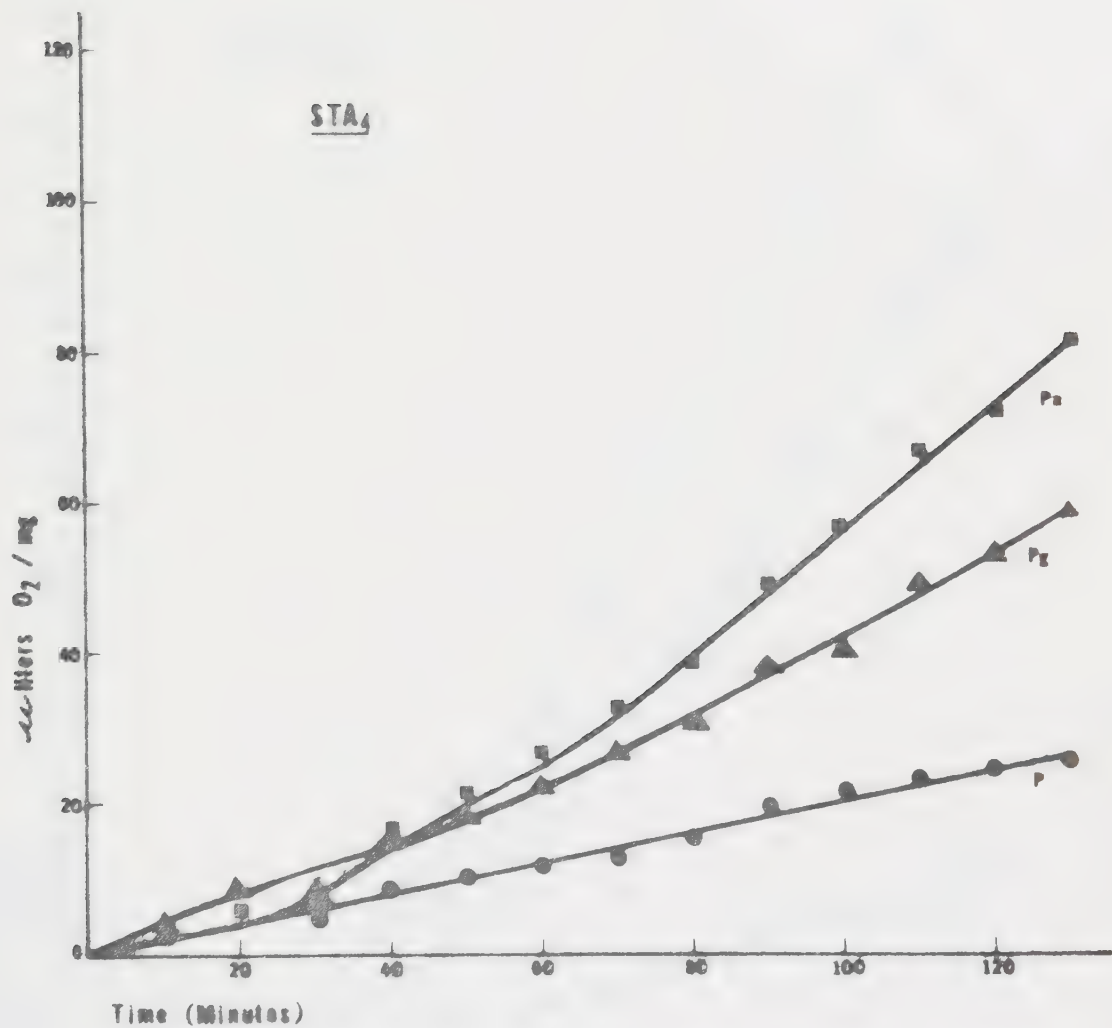


Fig. 15. Oxygen consumption by wild type conidia (STA₄)

Symbols: P, P-salts mixture alone; P_g, P-salts mixture with glucose; P_a, P-salts mixture with sodium acetate.

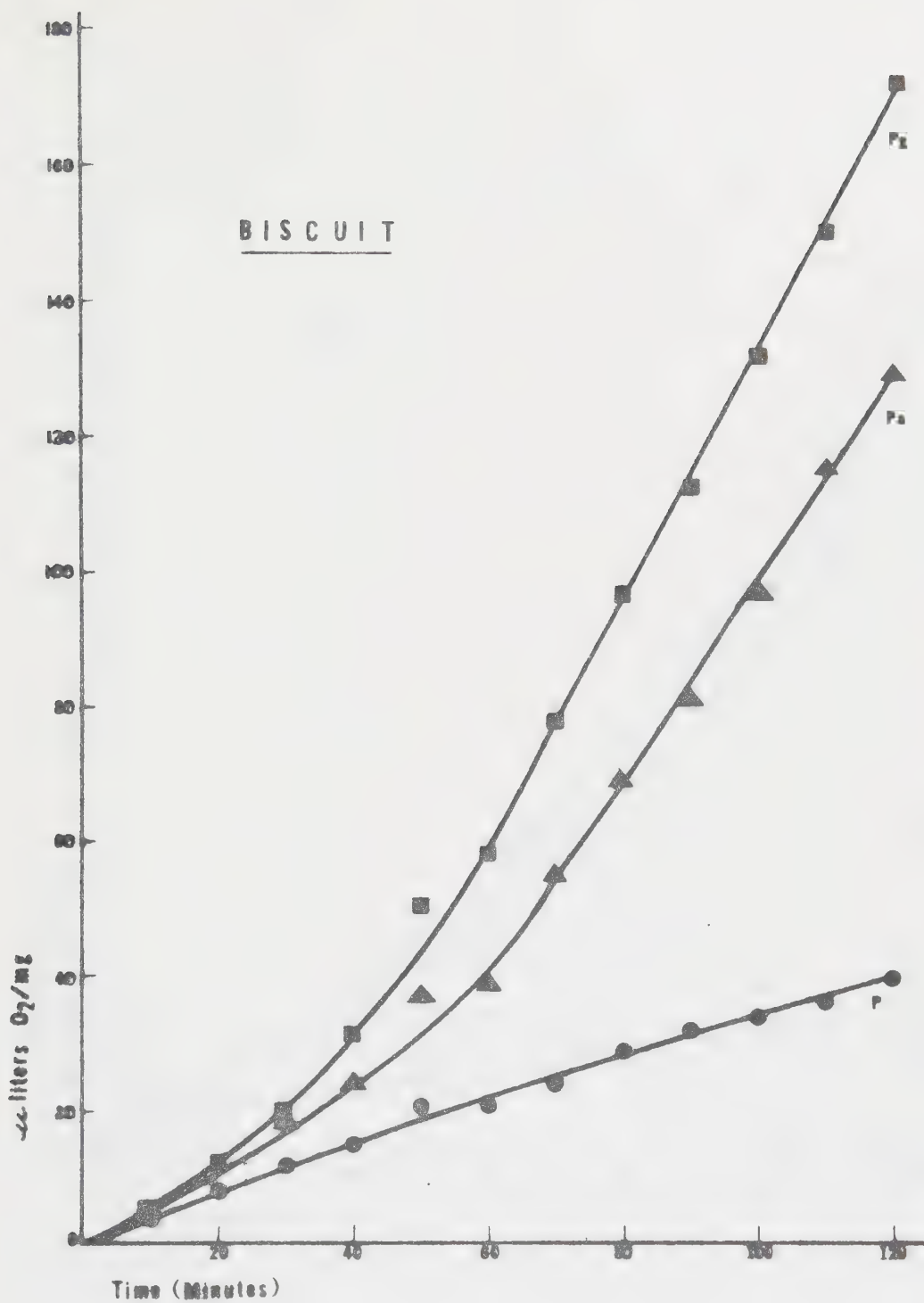


Fig. 16. Oxygen consumption by "biscuit"

Symbols: The same as those used in Fig. 15.

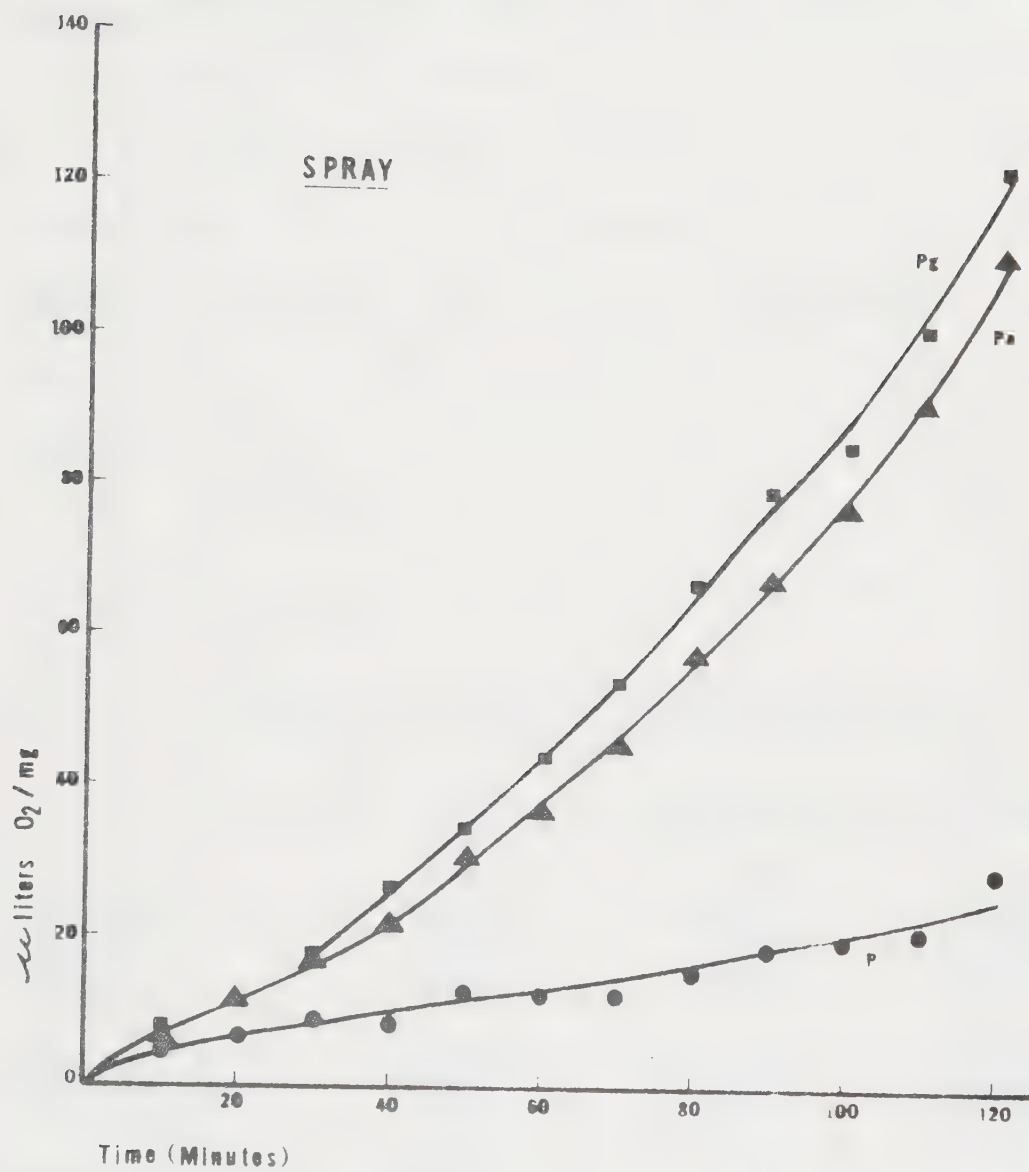


Fig. 17. Oxygen consumption by "spray"

Symbols: The same as those used in Fig. 15.

E. Partial Characterization of an Unknown Polymer Released into the Growth Medium of "Biscuit" and "Amycelial"

The search for a substance of this type was stimulated by the findings of Reissig (1968) who isolated a mucopolysaccharide (MP) from the culture medium of one of the colonial-temperature sensitive (COT) strains of Neurospora. MP was found not only to inhibit respiration in wild type conidia (DeBusk-unpublished results) but to produce colonial growth as well. This search led to the discovery of the polymer reported in this paper. Table 8 summarizes some of the

TABLE 8. A comparison of mucopolysaccharide from COT and the unknown polymers from "biscuit" and "amycelial"

	COT	amycelial	biscuit
mannose	.0366	.0339	.0388
galactose	.0270	.0347	.0447
glucose	.0715	.0576	.0470
% hexose	2.435%	2.30%	2.38%
amino acid	0.3375	0.1691	0.1584
% amino acid	3.54%	1.77%	1.663%
galactosamine	3.75	0	0
%galactosamine	60.456%	-	-

Values are given in micromoles per mg.

similarities and differences between an MP sample and the samples obtained from "biscuit" and "amycelial". As can

be seen from the table, these substances are quite similar with respect to their sugar and amino acid components. It should be mentioned that both the "biscuit" and "amycelial" substances are unable to influence either respiration or wild type colony morphology. It should also be noticed that galactosamine is completely lacking in the "biscuit" and "amycelial" samples.

Since less than 5% of the unknown substances could be accounted for in terms of sugars and amino acids, a sample of the "biscuit" substance was further analyzed for the presence of polyphosphate. The acid labile phosphorus was determined after hydrolysis with 1N HCl for fifteen minutes at 100 degrees. This method is based on the conversion of the inorganic phosphate thus obtained to phosphomolybdate at pH 4.0 in acetate buffer and then the complex being reduced by ascorbic acid. Readings were made at 700 millimicrons and comparisons made to phosphate standards indicated that more than 50% of the unknown polymer is composed of polyphosphate.

In an effort to more clearly characterize the sample a portion was placed on Sephadex G-100 column eluted with 0.5 M NaCl. The column had been calibrated against a wide range of protein markers so that a rough approximation of

molecular weight could be made. The bulk of the polymer was found in a fraction corresponding to a molecular weight of 5,400.

IV. DISCUSSION

In this study a number of different lines of thought and experimentation were followed in an attempt to gain a better insight into the problem of morphological mutations in Neurospora crassa. Along with their unusual growth patterns these mutants are characterized by numerous cellular and biochemical derangements, thus complicating the problem of separating cause and effect. Undoubtedly other differences will be found to exist as new data accumulate.

Electron micrographs of the mutant "amycelial" showed that this phenotype was associated with abnormalities of the mitochondria, cell walls, and endoplasmic reticulum. Media were developed which corrected to a large extent the abnormal growth patterns by favoring a more oxidative type of metabolism. This resulted in the induction of conidiation in several of the morphological mutants. Addition of certain sugars to these media greatly altered their capacity to modify the mutant phenotypes, and in several cases abolished it completely, the resultant growth being indistinguishable from that on minimal medium. Electron micrographs of "corrected" cultures of "amycelial" showed modifications of the mitochondria, cell walls, and endo-

plasmic reticulum to a condition more nearly resembling wild type cells. Further work with several of the morphological mutants showed disturbances occurring in respiration associated with high ethanol production, alterations in the free amino acid pools, and the accumulation of an unknown substance largely localized in the mitochondria of several of the mutants.

It seems likely that the immediate cause for the abnormal growth patterns is due to an altered cell wall composition. This is indicated by the release of the polyphosphate-containing polymer which is likely a normal or an altered cell wall component. As to the ultimate cause of this disturbance, only speculations are possible at this time since little is known of cell wall fine structure and even less of the control of its construction. The data would suggest that the mitochondria are involved either directly or indirectly in some way with this process in Neurospora.

It is interesting to note that many of the observations reported here have been observed in certain cancer cells. The high rate of aerobic glycolysis has its analogy in the high rate of lactic acid production in cancerous

cells. Other similarities include alterations in the endoplasmic reticulum and mitochondria. Certainly altered cellular morphology is a common property.

An alteration of cellular membranes has been forwarded by Wallach (1968) as a possible common explanation for these pleiomorphic effects in cancer cells. He cites the following data pertaining to neoplasia:

a. Endoplasmic reticulum -- The endoplasmic reticulum of neoplastic cells is markedly different from that of normal cells. The earliest morphologic aberrations during hepatic carcinogenesis are disorganization of the E.R., a disappearance of rough E.R., appearance of free polysomes, and a loss of glycogen granules.

b. Mitochondria -- Most normal tissues sharply reduce lactic acid production when there is sufficient oxygen available for oxidative phosphorylation (Pasteur effect). This regulatory mechanism is commonly defective in tumors apparently because of mitochondrial malfunction. Thus , tumors with high aerobic lactate production tend to have few and/or morphologically abnormal mitochondria.

In continuing the article, Wallach (1968), describes the two feedback systems linking glycolysis with oxidative

phosphorylation. Both involve obligatory intermediates. NAD/NADH and ADP/ATP, neither of which can freely permeate the mitochondrial membrane. The translocation of the reducing equivalents of NADH from cytoplasm to mitochondria is commonly abnormal in tumors. In many instances this involves impaired activity of the acetoacetate-beta-hydroxybutyrate "shuttle" of the mitochondrial membranes. Similarly, since ADP levels control oxidative as well as glycolytic phosphorylation, the defective control of glycolysis by the mitochondria of many tumors could be related to an abnormal distribution of this intermediate among the compartments of the tumor cell. Neither ADP nor ATP can cross the inner mitochondrial membrane by diffusion rather, permeation appears to involve specific enzyme systems located in the inner mitochondrial membrane. Thus, high, aerobic glycolysis, the most widely reported metabolic aberration of tumors, may relate to defects in membrane mediated control mechanisms.

In discussing the subject of the transfer of reducing equivalents it should be mentioned that any substance that can be reduced extramitochondrially by a pyridine-nucleotide-linked reaction and yields a product that is a substrate

for mitochondrial oxidation can serve to transfer reducing equivalents. Among these, the glutamic dehydrogenase (GDH) system is of particular interest as a possible pathway. Although apparently this pathway does not function in this way in mammalian systems (Boxer and Devlin, 1961), this does not rule out the possibility in Neurospora.

Whether the glutamic dehydrogenase system of Neurospora has these properties or not, it should be considered as a subject for future work. It will be recalled that the basis for the nutritional screening of morphological mutants was the inability of the mutants to utilize glutamic acid as sole source of nitrogen in an acetate containing medium. Since wild type cells grow well on this combination it would seem profitable to investigate the glutamic dehydrogenase system, probably the first step in the utilization of exogenously supplied glutamic acid under these conditions.

Kapoor and Smith (1968) described several interesting mutants of Neurospora (am-morphs) in which a single mutational event lead to the loss of NADP glutamic dehydrogenase activity, a loss of the inductive effects of amino acids on NAD-glutamic dehydrogenase and the appearance of a semicolonial growth habit in which conidiation was greatly diminished.

The hypothesis that a single mutational event was responsible for these changes was substantiated by the isolation and analysis of a spontaneous revertant having normal mycelial morphology and glutamic dehydrogenase activity.

Also implicating glutamic dehydrogenase in some way with morphogenesis is a study with Schizophyllum commune (Dennen and Niederpruem, 1967) where a pattern of variation was revealed in the levels of NAD-GDH and NADP-GDH in the cells during different phases of development.

In the present work a wide variety of observations were made. A few possible explanations were forwarded and a tentative direction for future work was indicated. As new data become available however, these ideas will probably require modification since the entire problem of morphological mutations in fungi is in its infancy.

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V. SUMMARY

Casamino acids, utilized in a growth medium as sole source of both carbon and nitrogen strikingly changed the morphology of wild type and several morphological mutants of Neurospora. Some of the changes that were seen include; (1) loss of colonial characteristic; (2) stimulation of conidiation (even in a strain previously thought aconidial); (3) modification of mycelial branching; (4) pigment changes. Certain other morphological mutants responded weakly or not at all to the casamino acids media. Of the group which did respond however, addition of certain sugars negated partially or completely the effect of the casamino acids while other carbon sources such as pyruvate, acetate, and Krebs cycle intermediates had no effect. Electron micrographs of the morphological mutant "amycelial" revealed important modifications of the cell wall, endoplasmic reticulum, and mitochondria accompanying an induction of conidiation using the casamino acids system.

The problem was followed using these data. The morphological mutants were further found to differ from wild type strains in several ways. The mutants were

found to possess lower free amino acid pools, abnormal rates of respiration and elevated production of alcohol, similar in these respects to the M-cultures of Turian (1964) where conidiation was suppressed in wild type strains by favoring this type of metabolism.

In addition, nutritional differences were found to exist between wild type and morphological mutant strains; an observation that led to a general method for the isolation of large numbers of these mutants.

Using the mutant strains "amycelial" and "biscuit", analyses of mitochondrial extracts revealed the presence of an unknown ninhydrin-positive substance located primarily in the mitochondria of the mutants and largely lacking in similar wild type fractions.

Analysis of the growth media of "amycelial" and "biscuit" led to the identification of an antigenic polymer largely composed of polyphosphate associated with the abnormal morphological growth in these mutant strains.

RESUME

Les casamino-acides introduits dans un milieu de croissance synthétique comme seules sources simultanées de carbone et d'azote ont modifié profondément la morphologie du type sauvage et de plusieurs mutants morphologiques de Neurospora crassa. Parmi les modifications observées, il faut relever: (1) la stimulation de la conidiation chez le type sauvage et son induction chez une souche considérée comme aconidienne, (2) la perte du caractère colonial de la croissance mycélienne chez divers mutants morphologiques, (3) un changement du type de bourgeonnement, (4) une intensification de la pigmentation. Certains mutants morphologiques n'ont cependant répondu que faiblement ou même négativement à ce traitement.

Chez le groupe qui a pu être modifié, l'addition de certains sucres, dont le glucose, a supprimé partiellement ou totalement l'effet conidiogène des casamino-acides (effet probable de répression catabolique). D'autres sources de carbone comme le pyruvate, l'acétate et des intermédiaires du cycle citrique n'ont pas eu d'effet suppressif. L'étude au microscope électronique du mutant

"amycelial" a révélé des modifications importantes au niveau des parois cellulaires, du réticulum endoplasmique et des mitochondries lors de l'utilisation des casamino-acides.

Ces premiers résultats ont été approfondis dans une deuxième partie. Sur le plan biochimique, il a été trouvé que les mutants morphologiques diffèrent du type sauvage en plusieurs aspects. C'est ainsi que ces mutants ne présentent qu'une faible teneur en amino-acides libres, un quotient respiratoire anormal et une production élevée d'éthanol. De plus, des différences nutritives ont aussi été mises en évidence entre le type sauvage et plusieurs mutants morphologiques. Leur incapacité à utiliser le glutamate ou l'aspartate en présence d'acétate a fourni l'occasion de proposer une méthode originale pour l'isolement sélectif de nouveaux mutants morphologiques.

Des analyses comparatives d'extraits mitochondriens des souches "amycelial" et "biscuit" ont révélé leur haute teneur en une substance inconnue (ninhydrine-positive) et qui n'existe qu'en traces dans les extraits similaires du type sauvage.

Enfin, dans les milieux de croissance des mutants

"amycelial" et "biscuit", un polymère antigénique a été découvert qui paraît associé à la croissance anormale de ces souches. Cette macromolécule se compose de sucres, d'amino-acides et de phosphate.

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